Biotechnology applied to the development of vaccines

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ONE OF the impacts of the modern biotechnology revolution has been a significant shift in the way we think and develop new vaccines. These changes reflect advances in the discovery of new antigens, adjuvants, vectors and delivery systems. While many of the vaccines currently administered to children and adults are still the result of methodologies developed in the mid-twentieth century, it is expected that the next few years will bring a growing number of new safer and more effective vaccines generated from genetic manipulation techniques and the production of recombinant proteins in heterologous systems. In this brief report, we will discuss some aspects of this change in practices and knowledge applied to vaccine development. In particular, we will emphasize research into the generation of therapeutic vaccines for tumor control, and their application to tumors induced by human papilloma virus.

The history of vaccines and their application in the prevention of infectious diseases spans over 200 years of dedication and hard work. Initiated by the brilliance and focused empiricism of physicians and researchers like Edward Jenner and Louis Pasteur, this area provides a beautiful example of reductionism applied to medical practice. Since the first vaccines based on pathogens - whether bacteria or viruses, attenuated or inactivated, very reactive and, in some cases, little efficient - vaccine research has moved towards the use of increasingly smaller fractions of these pathogens in the quest to increase safety without compromising efficacy. Thus, it is common to classify vaccines into three major groups (or generations) according to the strategies or concepts used in the preparation of the active principle, the vaccine antigens. First generation vaccines are those that include the entire pathogen, but subjected to treatments that lead to inactivation or attenuation of microorganisms. Also worth noting in this category is the strategy in which non-pathogenic microorganisms derived from a different host are used as antigens for vaccines aimed at the control of diseases caused by different but genetically-similar pathogens. A good example of this approach are smallpox vaccines based on vaccinia virus isolated from bovine cattle and TB vaccine that also uses a bacterium originally obtained from bovine cattle, the *Mycobacterium bovis* (BCG). Other highlights in the group of first generation vaccines include vaccines against whooping cough or pertussis

(vaccine cell), smallpox, polio, measles, rubella, and adenovirus among others.

The second vaccine generation emerged based on the idea that, for some pathogens, protection can be achieved after the induction of antibodies directed to a single target, such as a toxin, responsible for the disease symptoms, or surface sugars that allow the host immune system to neutralize and destroy bacteria that, otherwise, would spread rapidly before being noticed by our main immune defense lines. In this group, the highlights are acellular vaccines that use toxoids (toxins inactivated and purified by chemical treatments), purified proteins such as the vaccines against tetanus, diphtheria, and hepatitis B and polysaccharides, such as thoseintended to control meningococcal meningitis and pneumonia.

Finally, the third and newest generation of vaccines is based on an innovative concept that distinguishes it radically from the previous vaccine generation. These third generation vaccines use the genetic information of the pathogen responsible for encoding antigens relevant for protection. Generally called DNA or genetic vaccines, third generation vaccines were discovered empirically in the early 1990s in trials initially focused on the search of gene therapies in which genes are employed to replace defective genetic information originally present in the individual affected by certain genetic defects.

The concept of vaccine stemmed from the observation that animals inoculated with plasmids carrying genes expressed in transfected cells, i.e., cells in which the injected DNA has managed to penetrate the cytoplasmic and nuclear membranes and, subsequently, use the enzymatic transcription machinery required for translation of the antigen, which will trigger a series of immune responses, such as antibodies, essential to ensure protection against pathogens circulating in the bloodstream, cytotoxic cells, with the potential to identify and destroy infected cells even in the absence of circulating pathogens, and immunological memory, which is critical for a long lasting prophylactic effect. Although the initial perspectives for DNA vaccines was frustrated by the poor immunogenicity of several vaccines submitted to clinical trials, the results indicate that these vaccines can be excellent tools for the activation of cytotoxic immune responses and thereby for the control of intracellularly replication pathogens, such as viruses, some bacteria and certain types of cancers.

The advent of modern biotechnology, in particular the spread of genetic manipulation techniques, has changed in different ways the research and development of vaccines, whether of first, second or third generation vaccines. Through gene cloning and mutagenesis strategies we can accurately and more safely generate attenuated microorganisms (viruses and bacteria). Empirically isolated attenuated pathogens, used in first generation vaccines, can revert to the virulent state. Since in many cases the nature of the genetic change experienced by the microorganism during the attenuation process is unknown, the possibility of reversion to virulence, although unlikely, is a real. The techniques currently available for genetic manipulation enable selection , with relative ease,

of attenuated mutants in which genes involved with pathogenicity or primary metabolism are inactivated so as not to compromise the viability of the organism, but rather to make it incapable of causing diseases. However, the high costs of clinical trials and the established use of certain vaccine formulations such as the polio, measles, yellow fever viruses and the bacterium *Mycobacterium tuberculosis*, among others, reduce the interest of industries and laboratories to produce or develop new vaccine formulations (Table 1).

Biotechnology has revolutionized second generation vaccines. In an initial phase, these vaccines were restricted to the use of inactivated toxins, such as in the tetanus and diphtheria vaccine, and in a second phase, purified polysaccharides. The initial use of purified proteins from viruses or bacteria was limited to situations in which it was possible to cultivate and purify specific antigens, such as certain bacterial toxins, or obtain them from the serum of infected patients, as in the case of the hepatitis B virus. With the improvement of recombinant protein production techniques by heterologous expression systems, such as those based in bacteria, yeasts, insect and mammalian cells, are used as sources for antigens to be incorporated into vaccine formulations. In fact, this area of vaccinology based on the generation of subunit vaccines using recombinant antigens currently receives greater interest and attracts much investment due both to safety and financial return is, such as illustrated by the vaccine to control hepatitis B and, more recently, the preventive vaccine against infections with human papillomavirus (HPV) (Table 1).

DNA vaccines emerged as a result of biotechnological advances in recombinant DNA. The genetic information encoding antigens with vaccine application is cloned and propagated in *Escherichia coli* strains, a harmless inhabitant of our intestinal microbiota. The production procedure is relatively simple and less costly than that involved in obtaining recombinant proteins. Moreover, some features of the immune response modulation mediated by DNA vaccines have become a valuable tool in the development of therapeutic formulations. Undoubtedly, more than conventional preventive vaccines, DNA vaccines represented an alternative approach to develop and implement immunotherapies which became possible thanks to the introduction of recombinant DNA techniques in vaccine research (Table 1).

Vaccines with therapeutic properties

Therapeutic vaccines aim to control chronic infections or degenerative diseases affecting the individual to be treated. In this respect, the definition of a therapeutic vaccine superimposes with the concept of gene therapy, particularly, when one makes use of DNA vaccines or live vectors that ultimately transfer new genetic information into the host's cells. In the case of DNA vaccines the administration of the heterologous gene will be responsible for producing the protein that ultimately will trigger an immune response capable of reversing the established infectious or degenerative process. The goal of any therapeutic vac-

cine is therefore to reverse situations in which the individual's immune system was not able to activate an immune response with the appropriate intensity or quality, leading to the establishment of an immunological tolerance process. Obviously, the relevance of the therapeutic vaccine concept makes sense only in cases where more efficient therapeutic options are unavailable. Moreover, for a therapeutic vaccine to be successful, there must be evidence that the disease and, therefore, the pathogen that caused the disease can be controlled by the individual's immune system, once properly activated, as in the case of chronic diseases such as those caused by the human immunodeficiency (HIV), human herpes (HSV), hepatitis B (HBV) and human papilloma (HPV) viruses.

Advances in the knowledge of immunity mechanisms have enabled definition of important parameters for a therapeutic vaccine. In particular, the understanding of the importantrole of functional cytotoxic T lymphocytes, as well as of other immune system cells with similar functions, has represented an important step in the advancement of therapeutic vaccines. By recognizing and destroying cells, either infected or altered in their antigenic composition, CD8+T cytotoxic lymphocytes are a formidable barrier against pathogens that evade other immune defense mechanisms, such as antibody and complement, once the pathogen penetrate and multiply inside the host's cells.

In recent years, several research groups have been devoted to the search for vaccine formulations that can activate, in an efficient and long lasting way, CD8+T cytotoxic lymphocytes capable of recognizing and destroying cells expressing on their surface fragments of antigens derived from viral, bacterial or parasite pathogens observed after infection. Similarly, vaccines with anticancer properties have been investigated for several decades in the hope of finding more effective and less invasive alternatives for the treatment of some types of cancer. In some cases, the cancer treatments may become more favorable for a therapeutic vaccine approach, like cancers linked to an infectious origin, such as tumors associated with infection with HBV and HPV. In both cases, the availability of vaccines with prophylactic properties aimed at the control of infection with viruses makes the issue of viability and relevance of therapeutic vaccines more complex and, in certain situations, generates interesting controversy about the potential role of prophylactic or therapeutic vaccines in the control of the different cancer diseases. In this respect, the recent marketing of prophylactic vaccines for HPV infection and the search for alternative vaccines with therapeutic properties against tumors associated with infection by this virus represents an example that deserves investigation.

Table 1 – Main contributions of biotechnology to vaccine development

Vaccine type	Biotechnological strategy	Vaccines
Sub-unit vaccines	Production of recombinant proteins in heterologous systems	Hepatitis B, acellular pertussis, HPV
Bivalent attenuated pathogens	Genetic manipulation for insertion of genes encoding antigens	Dengue fever,* BCG,* Salmonella Typhi,* Adenovirus*
DNA vaccines	Immunization with recombinant plasmids	Melanoma vaccine #

* Vaccines not yet available for use in humans; # vaccine for use in dogs.

The search for a therapeutic vaccine to control tumors associated with HPV infections

Currently, it is well established that the human papilloma virus (HPV) is the main etiological agent of cervical cancer, as well as of other cancers such as anogenital and head and neck tumors. In relation to cervical cancer, the HPV virus is associated with virtually all cases observed. This type of cancer is of high epidemiological relevance, as it represents the second leading cause of death by cancer among women worldwide, causing approximately 270,000 deaths per year, with about 500,000 new cases diagnosed annually (Zur Hausen, 2009).

More than 100 types of HPV have been described and can be classified according to their oncogenic potential. Some types of HPV can cause low-grade genital warts and benign lesions, and are called low-risk genotypes, among which the most common are HPV-6 and HPV-11. HPV types associated with the development of cervix tumors are called high risk and the most frequently found are HPV-16, HPV-18, HPV-31, HPV-33 and HPV-45. HPV-16 and HPV-18 are the types most commonly associated with cervical cancer, totaling 75 percent of the incidence of cases of this cancer, with HPV-16 being the most common in Brazil and in most countries (Bosch et al., 1995). Consequently, these two types of HPV, particularly HPV-16, have been used as a model for the development of vaccine, either prophylactic against virus infections or therapeutic aiming tumor erradication.

Understanding the molecular biology of the HPV virus is essential for vaccine development. HPVs are non-enveloped double-stranded DNA viruses. The genome of this virus encodes two late expression proteins, L1 and L2 ("L" from late), which form the viral capsid, and six proteins with regulatory functions: E1, E2, E4, E5, E6 and E7 ("E" from early). In particular, the E6 and E7 proteins of high-risk HPVs act on cell maligninization through binding or inactivation of the products of tumor suppressor genes, p53 and pRB, respectively (Dyson, 1998). Thus, cells infected with high-risk HPVs can develop genomic instability and uncontrolled replication that can progress to cancer.

As cervical cancer is caused by a viral infection, there is a expectation that a vaccine capable of generating neutralizing antibodies directed against the viral capsid proteins L1 and/or L2 will block the virus entry and, thereby, reduce the incidence of cancer in the long term. Two prophylactic vaccines based on VLPs (Virus Like Particles) formed by the L1 proteins of HPV-6, -11, -16, and 18 (Gardasil) or HPV-16 and 18 (Cervarix) are available on the market. These vaccines have proven to be extremely efficient at inducing neutralizing antibodies to the virus. It is expected, therefore, that individuals immunized with these vaccines will be protected against infection with the virus types used in the vaccine preparation and, therefore, will not develop cervical cancer in the future. However, because of large natural variability, immunity is specific for the viruses used to generate VLPs.

Women who have high-grade lesions and HPV-induced tumors do not produce viral particles and therefore would not benefit from the VLP-based vaccine strategy. By penetrating the epithelial cell, the virus integrates into the cellular genome and stops replicating. The synthesis of L protein is completely blocked, but the synthesis of some regulatory proteins, in particular the E6 and E7 oncoproteins, remains unchanged. These cases require the search for vaccines aimed at controlling HPV-induced lesions. These vaccines with therapeutic anti-tumor properties should favor the induction of cytotoxic responses, so as to lead to the recognition and killing of infected cells. The main targets for this type of vaccine are the E6 and E7 proteins, constitutively expressed in cervical carcinoma cells.

There are no therapeutic vaccines for HPV-induced tumors available on the market, but several formulations are being investigated in animal models and some are already in the clinical trial phase. Several approaches have been used for the development of a vaccine with these characteristics, such as those based on synthetic peptides, purified proteins, viral or bacterial vectors and even dendritic cells or tumor cells, among others. In this context, DNA vaccines emerge as an interesting strategy for the generation of antigen-specific immune responses which, for their ability to induce cytotoxic responses, would act only on tumor cells, avoiding such side effects as those observed in current radiation and chemotherapy treatments.

Some groups engaged in the research of DNA vaccines for the control of HPV-induced tumors are seeking to increase the immunogenicity of vaccine formulations. In this context, the use of adjuvants or constructions that express hybrid proteins in which HPV oncoproteins are fused to antigens that enhance immunogenicity for cytotoxic responses has received special attention. A promising alternative is represented by DNA vaccines encoding HPV proteins genetically fused to the herpes simplex virus type I (HSV) glycoprotein (gD). The gD protein has a transmembrane region, and thus the HPV-16 oncoproteins fused thereto are directed to the membrane of transfected cells, thereby drastically re-

ducing the risk of interaction with cell cycle proteins and exposing them to the immune system in a more efficient way.

Other adjuvant roles assigned to the gD protein have been described and are associated with the ability to bind to the HVEM receptor (Herpes Virus Entry Mediator), belonging to the family of tumor necrosis factor receptors (TNFR). Through interaction with this receptor, it was demonstrated that the gD protein generates stimulus signals to cells of the immune system by activating a transcription factor, NF-kB (Cheung et al. 2009). Additionally, the gD protein increases the immunogenicity of proteins fused thereto by competing with another membrane protein called BTLA (B and T Lymphocyte Attenuator), an inhibitory factor that suppresses the activation of B and T lymphocytes by antigen-presenting cells, for the same binding site that the two proteins have on another cell receptor called HVEM (Herpes Virus Entry Mediator). As a final result, cells expressing the gD protein on the surface reduce the inhibitory effects of BTLA and promote a more efficient activation of B and T lymphocytes (Lasaro et al. 2008).

The use of DNA vaccines encoding HPV-16 oncoproteins genetically fused to HSV-1 gD protein has been proposed by our group as a strategy for controlling HPV-16-induced tumors (Lasaro et al. 2005). These vaccines have been tested in mice for their ability to activate antigen-specific $CD8^+$ T cells and prevent the development of tumors after implantation of epithelial cells modified to express the E6 and E7 proteins of HPV-16, called TC-1 cells. Animals immunized with these vaccines showed induction of E7-specific $CD8^+$ cells and a 40 percent therapeutic antitumor effect against the development of tumors after receiving four doses of the vaccines. In these experiments, tumor cells are implanted into the animal, followed by treatment with the DNA vaccine, thus characterizing the therapeutic vaccine effect.

The vaccine strategy against HPV-16-induced tumors has been improved by the construction of a new DNA vaccine expressing the HPV-16 E5, E6 and E7 oncoproteins genetically fused to HSV-1 gD (Diniz et al. 2010). Experiments designed for checking the activation of $CD8^+$ T cells specific for HPV-16 oncoproteins showed that a single dose of the vaccine was sufficient to generate significant activation of E6 and E7-specific $CD8^+$ cells (Figure 1A). The vaccine was able to generate 100 percent protection against tumors in animals that were first immunized and subsequently challenged with TC-1 cells. The antitumor effect of the vaccine was associated with the involvement of $CD8^+$ T cells, but not of $CD4^+$ T cells, in trials in which these cell populations were differentially removed with antibodies (Figure 1B). Moreover, this vector conferred 70 percent protection in mice challenged with TC-1 cells and subsequently immunized with three doses of the vaccine.

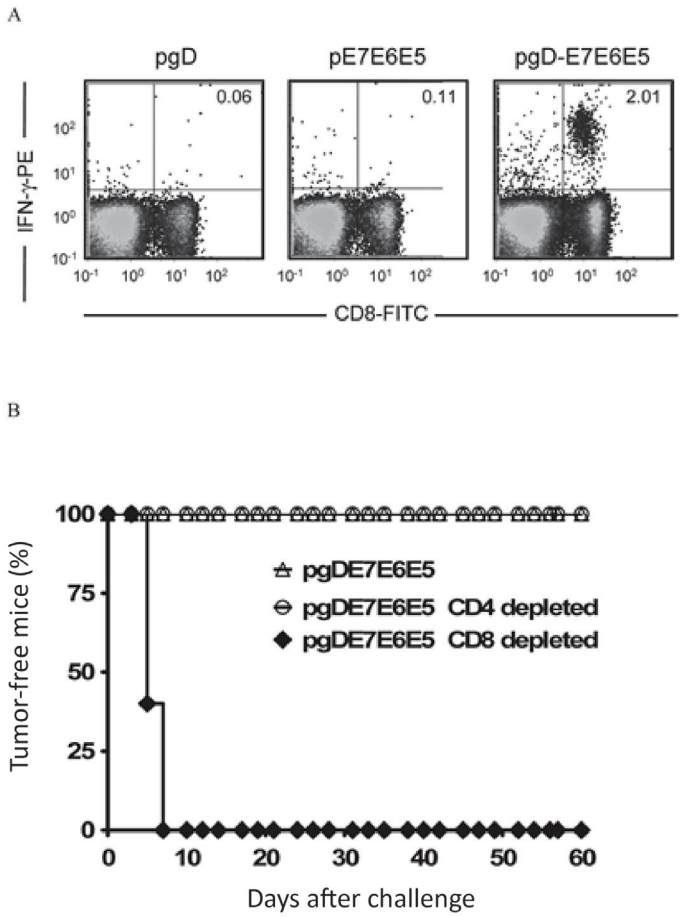

Figure 1 – Activation of E7-specific CD8+ T cell precursor and preventive protection against tumors induced by TC-1 cells in mice immunized by parenteral route (intramuscular) with the pgD-E7E6E5 vector. (A) Activation of CD8+ T cell precursors in mice i.m. immunized with one dose of the pgD, pE7E6E5 or pgD-E7E6E5 vectors (100 µg / dose). The percentages of CD8+ IFN-γ+ / CD8+ total cells were determined two weeks after the last dose of the vaccine. Detection of E7-specific CD8+ T cells was performed with PBMC stimulated with synthetic peptide derived from E7 protein (49RAHYNIVTF57) specific for MHC-I and stained with anti-CD8 (FITC) and intracellular IFN-γ antibodies (PE). (B) Vaccine induced preventive protection against the development of tumors in mice immunized with one dose of pgDE7E6E5 and depletion of CD4+ or CD8+ cells through inoculation of specific antibodies. The mice were challenged with 5x105 TC-1 tumor cells two weeks after administration of the vaccine.

Cytokines related to the activation or proliferation of cells of the immune system can be used as an alternative strategy to increase DNA vaccine-induced

response. Based on previous studies, we have developed a third version of DNA vaccines against HPV-16-induced tumors, which uses the co-administration of plasmids expressing the IL-12 or GM-CSF cytokines in combination with the plasmids expressing HPV-16 E7 or E7E6E5 genetically fused to HSV-1 gD. The use of this combined immunization system enabled achieving a maximum therapeutic protective effect, i.e., 100 percent tumor-free mice, with the co-administration of the plasmid expressing IL-12 or GM-CSF and the plasmid expressing the target antigen (Figure 2). Markedly, the increased antitumor effect of the vaccine enabled reducing the number of vaccine doses required for the complete elimination of the tumors, and with a single dose it was possible to confer therapeutic protection to all animals implanted with tumor cells.

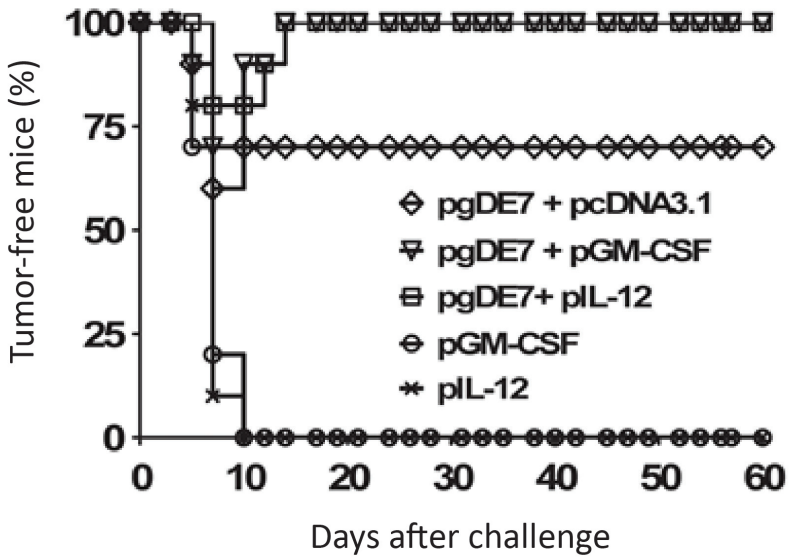

Figure 2 – Co-administration of pgDE7 vector and plasmids encoding IL-12 or GM-CSF confers therapeutic protection to challenges of TC-1 cells. The mice were immunized with three doses (100 µg) of pgDE7 combined with 100 µg of pcDNA3.1 (empty vector), PGM-CSF or pIL-12 vectors. The vaccines were administered 8 hours after challenge with 5 x 10_5 TC-1 cells. Tumor growth was monitored up to 60 days after challenge.

The evidence generated by these studies demonstrate that the proposed vaccine strategy is very promising as an immunologic alternative for the control of HPV-16-induced tumors, with results better than those reported in the literature for other formulations tested in animal models. Additional studies are being carried out with the aim to test such vaccine formulations in clinical conditions. For this purpose, additional modifications will be necessary to allow increased immunogenicity in humans and decreased inflammatory reactions from the use of material obtained under good production conditions.

Final considerations

Biotechnology has contributed decisively to the improvement of processes related to the development and production of new vaccines or to the improvement of existing vaccines, for these to become safer and more effective. The availability of prophylactic anti-virus vaccines and the perspective for the development of vaccines with therapeutic effect on HPV-associated tumors clearly illustrate the impact of modern biotechnology on the field of vaccine research. Epidemiological data released by the National Cancer Institute (INCA) show that in Brazil approximately 20 out of each 100,000 women develop cervical cancer. A total of 20,000 new cases are diagnosed each year, an incidence twice as high as that recorded in more developed countries. These data highlight the impact that vaccines directed to the control of HPV-associated tumors may have in the country.

The availability of prophylactic vaccines against infection with two HPV types has created great expectation towards a future reduction in the number of lives lost to the disease, as well as a decrease in the economic costs associated with the treatment of people with lesions in more advanced stages of this type of cancer. Reality, however, shows that the greatest impact of the vaccine formulations introduced in the market has been of an economic nature for the pharmaceutical companies. The high cost of the vaccine (approximately $1,000.00 per person) has enabled manufacturers to earn profits exceeding $3 billion annually, a fact that has awaked the interest of many laboratories in investing in vaccines capable of yielding high financial returns. In turn, the real contribution of these vaccines to reduce the impact of the disease on the world is still unknown, since the people presently being capable to afford for them are less likely to feel the more serious consequences of the disease.

The development of a vaccine with therapeutic properties for the control of cancer associated with HPV infection would bring interesting perspectives in terms of real benefit of disease control. Unlike prophylactic anti-viral vaccines, therapeutic anti-tumor vaccines are expected to confer protection to a variety of HPV types and prevent the appearance of tumors in individuals already infected. The availability of this kind of treatment to the public health system, once the efficacy could be demonstrated in clinical trials, can have an immediate impact on the reduction of the number of deaths associated with the disease, and dramatically reduce the cost and traumas associated with the treatment of advanced cancer cases. However, as all research at an early stage of development, the allocation of resources necessary to demonstrate clinical effects will be essential for these perspectives to materialize.

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Abstract – Vaccines represent the intervention strategy with the best cost-benefit ratio so far applied in public health. Biotechnological advances in various areas of vaccine research have contributed to the development of safer and more effective formulations. Moreover, application of biotechnology tools to vaccine development has caused changes in the way we think and produce these reagents for use in both humans and animals. Such technologies bring renewed perspectives that, in the near future, vaccines for the control of several non-preventable infectious and degenerative diseases will be available. In particular, the development of vaccines with therapeutic effects, although representing a huge challenge, are getting closer to reality and will have a tremendous impact on the treatment of several diseases such as some cancer forms.

Keywords: Vaccines, Biotechnology, HPV, Cancer, Therapeutic vaccines.

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